Genetic immunization with the immunodominant antigen P48 of Mycoplasma agalactiae stimulates a mixed adaptive immune response in BALBc mice

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Abstract

A DNA vaccine against contagious agalactia was developed for the first time, encoding the P48 of Mycoplasma agalactiae. Specific immune responses elicited in BALB/c mice were evaluated. Both total IgG and IgG1 were detected in mice vaccinated with pVAX1/P48. Proliferation of mononuclear cells of the spleen, levels of gamma interferon, interleukin-12, and interleukin-2 mRNAs were enhanced in immunized animals. Results indicate that pVAX1/P48 vaccination induced both Th1 and Th2 immune responses. Nucleic acid immunization could be a new strategy against M. agalactiae infections and may be potentially used to develop vaccines for other Mycoplasma diseases.

Keywords Genetic immunization; DNA vaccines; Mycoplasma agalactiae; Artificial active immunity

1. Introduction

Contagious agalactia (Ca) of small ruminants caused by Mycoplasma agalactiae is a serious disease affecting mainly sheep and goats characterized by mastitis, polyarthritis, keratoconjunctivitis, and abortion (Cottew, 1979, Da Massa, 1983 and Bergonier et al., 1997). The disease occurs in Europe, Western Asia, the United States of America (USA), North Africa, and is endemic in the Mediterranean Countries (Bergonier et al., 1997). Ca causes severe economic losses in areas where economy is largely based on sheep farming, resulting from reduced milk production and increased lamb mortality (Lambert, 1987). Antibiotics inhibiting bacterial cell wall assembly, such us penicillins, are not effective against M. agalactiae, whereas tetracyclines and macrolides can sometimes bring about clinical improvements, but they result in the selection of unaffected carriers hosting antibiotic-resistant strains. Therefore, M. agalactiae infection is tentatively controlled through the implementation of hygienic measures, and through laboratory monitoring of flocks/herds with replacement of infected animals (Nicholas, 2005). However, in addition to improved hygienic and animal management procedures, the effective prevention of contagious agalactia requires vaccination.

Both attenuated and inactivated vaccines against M. agalactiae have been used with mixed success (Gil et al., 1999 and Nicholas, 2005). Despite their intrinsic disadvantages, related to safety, difficulty of development and production, cost, transport and storage (Gurunathan et al., 2000), live attenuated vaccines have been used in Turkey for many years, and have been reported to provide better protection than inactivated vaccines. However they can produce a transient infection with shedding of mycoplasmas (Nicholas, 2002). In Europe, where the use of live vaccines is not allowed, attention has focused on the use of inactivated organisms. Those which have provided protection from clinical disease have done so to different extents (Tola et al., 1999 and Greco et al., 2002). However they induce mostly a short-lived

humoral immune response. Moreover, traditional vaccines can require multiple booster shots (inactivated vaccines), or hamper differentiation (inactivated and attenuated) of vaccinated from infected animals (DIVA). Nucleic acid immunization is a new promising approach to develop effective and safe marker vaccines suitable for DIVA diagnostics (Gurunathan et al., 2000, van Oirschot, 2001, Brandsma, 2006 and Laddy and Weiner, 2006). It has been shown that several DNA vaccines against mycoplasmas have the capacity to induce immune responses in the murine model (Barry et al., 1995, Lai et al., 1997 and Chen et al., 2003; March et al., 2006). Therefore, we speculated that a DNA vaccine could be also developed to control M. agalactiae infection.

Mycoplasma agalactiae, and mycoplasmas in general, trick the immune system either by phase and size variation of their surface antigens (Citti et al., 2000 and Glew et al., 2000) or by modulating the host immune responses through the expression of up- and down-regulating cytokines inducing suppression or aspecific polyclonal stimulation of B and T lymphocytes (Razin et al., 1998). Membrane surface proteins can also play a basic role in pathogenesis of mycoplasmas especially by acting as cytoadhesins in the attachment of mycoplasmas to host cells (Zhang et al., 1995 and Santona et al., 2002). Evasion of immune responses through surface antigen variability and modulation of immunity may render vaccination ineffective. We recently described the P48 of M. agalactiae as an invariable, constantly expressed, immunodominant, surface lipoprotein (Rosati et al., 1999) homologue to the MALP-404 lipoprotein of Mycoplasma fermentans (Davis and Wise, 2002). We also developed a commercial recombinant indirect ELISA for contagious agalactia (Institute Pourquier, France) based on the use of the P48 antigen lacking the leader peptide, which has been proven to be associated to high levels of specificity and sensitivity (Rosati et al., 2000).

Therefore, P48 was the selected gene to incorporate into a DNA vaccine against M. agalactiae, and its potential was evaluated in the present study by detecting and analyzing P48-specific antibodies, the proliferation of splenocytes, and the T cell-specific cytokines released in immunized BALB/c mice.

2. Materials and methods

2.1. Construction of expression plasmids

The mammalian expression vectors pcDNA3.1 (Invitrogen), pVAX1 (Invitrogen), pCMV-Script (Stratagene), and the bacterial expression vector pGEX-2T/P48 (Rosati et al., 2000), were chosen to clone and express the P48 antigen gene of M. agalactiae. Briefly, the 1.3 kb DNA fragment containing the P48 gene, lacking the leader peptide, was obtained by PCR (Mastercycler Gradient, Eppendorf) from plasmid pGEX-2T/P48, using the primer pair MagP48/BamHI/K/ATG/F (5′-CTGGGATCCACGATGGTAAAAACTATTTCAACACTTGCA-3′) and MagP48/ECO/R (5′-CACGAATTCTTATTTTCTTGTTTCAGAAGCCAA-3′). The reaction was carried out in a thermal cycler (Mastercycler gradient, Eppendorf) with the following profiles: initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30′′, 65 °C for 20′′, and 72 °C for 1.5 min, and a final extension step at 68 °C for 10 min. The DNA fragment was digested with BamHI and EcoRI, purified, and then inserted into pcDNA3.1, pVAX1, and pCMV-Script, predigested with BamHI and EcoRI, obtaining plasmids pcDNA3.1/P48, pVAX1/P48, and pCMV-Script/P48, as DNA vaccine candidates. pGEX-2T/P48 was used to express antigen for immunoblotting and ELISA.

2.2. Expression of P48 gene in transfected cells

The human embryonic kidney cell line HEK 293 was transfected with pcDNA3.1 or pcDNA3.1/P48, pVAX1 or pVAX1/P48, and pCMV-Scrip or pCMV-Script/P48, alternatively, using the CalPhos Mammalian Transfection

Kit (Clontech), according to the manufacturer's instructions. Transfected cells were harvested 48 h later and washed with phosphate-buffered saline three times. Total RNA was extracted with Trizol reagent (Invitrogen). Complementary DNA (cDNA) was obtained from RNA with the SuperScript™ First-Strand Synthesis System (Invitrogen). P48 cDNA was amplified by PCR using primers MagP48/BamHI/K/ATG/F and MagP48/ECO/R as described above. The amplified products were analyzed with a 1% agarose gel. Robustness of plasmids expressing P48 was evaluated by replicating the expression experiments three times and by immunoblotting.

2.3. Immunization of mice

Twelve six-week-old female BALB/c mice (Charles River Laboratories) were used according to the EU International Guidelines. Animals were isolated in individual cages and were injected intramuscularly (T1) both in the left and the right hind thigh muscle with either 50 μg (1 μg/μl) of pVAX1/P48 (vaccine group, six mice), or with 50 μg (1 μg/μl) of pVAX1 (control group, six mice), alternatively. Plasmid preparations were formulated in 50 μl of sterile PBS containing 0.5% bupivacaine-HCl. Each animal was boosted with the same dose 15 (T2) and 30 (T3) days after the first immunization. Serum samples, collected by tail bleeding before the first immunization (T0), and every 14 days for 8 weeks (T1, T2, T3, Tf), were analyzed for P48-specific antibodies by Western blotting (pooled sera) and by rP48 ELISA (each serum individually tested).

2.4. Expression and purification of P48 antigen

Escherichia coli strain JM105 was transformed with plasmid pGEX-2T/P48, and recombinant P48 was expressed and purified, as previously described (Rosati et al., 2000). Briefly, early log phase cultures of positive clones, induced for 2 h with 0.5 mM isopropyl b-thiogalactopyranoside (IPTG) were centrifuged and lysed by conventional physico-chemical methods. Recombinant fusion protein was recovered in the soluble fraction and purified by affinity chromatography. Thrombin cleavage was performed, and purity and yield of rP48 estimated by SDS–PAGE and Bradford method.

2.5. SDS–PAGE and western blot analysis

The purified rP48 protein was used in immunoblotting for testing the presence of specific antibodies in sera of vaccinated and control mice. Recombinant P48 and BenchMark™ Pre-stained Ladder (Invitrogen, Italia) were run on NuPage Novex 4–12% Bis–Tris Zoom Gels (Invitrogen) in the XCell SureLock Mini-Cell (Invitrogen) according to vendor instructions. Electrophoretic transfer onto nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech) was done with a mini-Trans-Blot electrophoretic cell system (Bio-Rad). The membrane was blocked by incubation with 5% skim milk in TBS for 1 h at room temperature. Mouse sera, diluted 1:100 in TTBS (TBS with 0.05% tween 20, and 1% skim milk) were added and incubated for 1 h at room temperature. The membrane was then washed with TTBS three times. Goat anti-mouse IgG antibodies conjugated with horseradish peroxidase (Sigma) were diluted 1:500 in TTBS (TBS with 0.05% tween 20, 1% skim milk) and used as secondary antibodies (1 h of incubation at room temperature). Again, the membranes were washed with TTBS three times and treated for detection with the CN/DAB substrate kit (Pierce). A rabbit hyperimmune antiserum was raised against P48, as previously described (Alberti et al., 2006), and used to detect expression of rP48 in transfected HEK 293 cells. Western blots were performed as described above and using peroxidase conjugate Protein G (Pierce) as a secondary antibody.

2.6. Assay for splenocyte proliferation and cytokine mRNA quantification

Mononuclear cells from the spleen, harvested and prepared from mice as previously described (Xiao et al., 2004), were plated in 96-well flat-bottom plates at 100 μl per well (2 × 105 cells per well). Subsequently 100

µl per well of medium with or without recombinant P48 (15 µg/ml) were added and mixed. Concanavalin A (5 µg/ml; Sigma) was used as a positive control.

Each splenocyte sample was plated in triplicate. In order to evaluate the specificity of the assay mononuclear cells were also incubated in two independent experiments with recombinant Mycoplasma Capricolum subsp. capricolum P60 (Alberti et al., 2007). The proliferative response was measured by Cell Titer 96 AQueus one solution cell proliferation assay, according to vendors instruction (Promega). Plates were read at 490 nm. The stimulation index (SI) was calculated as the ratio of average OD value of wells containing antigen-stimulated cells to average OD of wells containing only cells with medium. Complementary DNA was obtained from total RNA extracted from mice spleen with Trizol reagent (Invitrogen), by using the SuperScript™ First-Strand Synthesis System (Invitrogen). Pairs of oligonucleotide primers effective on mouse cytokines and Th transcription factors were chosen form the literature as follows: IFN-γ and IL-2 (Ramos-Payan et al., 2003); IL-12p40 and IL-4, (Liu et al., 2005); T-bet (Liu et al., 2003); gata3 (Ise et al., 2002). Traditional PCR was used to evaluate the selected oligonucleotides and establish the presence of different cytokines expression levels in vaccinated and control mice. The same primers were used in real time PCR experiments conducted to investigate the relative quantities of each cytokine/transcription factor in control and vaccinated groups. On the basis of traditional RT-PCR results, primers previously selected for IFN-γ were discarded in favour of new generated oligonucleotides (IFN-γ/MOUSE/F: 5'TACTGCCACGGCACAGTCAT 3', IFN-γ/MOUSE/R: 5' tccttttgccagttcctcca 3'). β-actin primers (Ramos-Payan et al., 2003) were used in traditional and real time PCR to amplify β-actin, selected as an internal control to normalize gene expression.

Real time PCR (7900HT Fast Real-Time PCR System, Applied Biotechnologies) amplifications were performed by using the Platinum SYBR Green qPCR Super Mix – UDG following vendor instructions (Invitrogen). Following an initial denaturation step of 2 min at 95 °C, forty cycles of PCR were performed with cycling conditions of 15 s at 95 °C, and 60 s at 60 °C. PCR signals were with the relative quantification DDCT option of the SDS 2.2.2 software, by using B-Actin mRNA as an internal control to normalize the levels of cytokine-specific mRNAs detected in different samples. Each experiment was repeated at least three times.

2.7. Antigen-specific ELISA analysis

The antibodies specific to P48 were analyzed. Briefly, microplates (Nunc maxysorp) were coated overnight uncovered at 37 °C with 100 ng/well of rP48. After four washes, plates were blocked with 150 µl/well of PBS containing 2.5% casein (Sigma), incubated at 37 °C for 1 h, and washed as before. Serum samples, diluted 1:20 in PBS containing 1.25% casein, were incubated at 37 °C for 2 h (100 µl/well).

Wells were then washed and incubated with 100 ng/ml peroxidase (HRP) labeled goat anti-mouse IgG (Sigma), goat anti-mouse IgG1-HRP (Santa Cruz), or goat anti-mouse IgG2a-HRP (Santa Cruz). After final washes, the enzymatic reaction was developed with p-nitrophenyl phosphate in 3,3',5,5'-tetramethylbenzidine solution (Zymed Labs), stopped after 5 min by adding 0.2 M sulphuric acid and read at 450 nm. Box plots of ELISA absorbances were obtained for mice belonging to control and vaccinated groups at each sampling time by Minitab.

2.8. Statistical evaluation

The significance of differences of medians and means between the two groups was evaluated with Minitab (Minitab release 13.0), by using the non parametric Mann-Whitney and the Anova tests, respectively. All

conclusions were based on significance levels of $P < 0.05$. Boxplots representative of results were generated by using the same software.

3. Results

3.1. Construction and expression of plasmid carrying P48 gene

Nucleic acid immunization has been reported to induce both humoral and cellular immunity in many infectious disease models (Lowe et al., 2006). Plasmids pcDNA3.1/P48, pVAX1/P48, and pCMV-Script/P48 were constructed in order to investigate the potential of a plasmid carrying the 48 kDa antigen gene of M. agalactiae in eliciting immune responses. To ensure that P48 could indeed be transcribed in mammalian cells, the presence of specific mRNA was established by RT-PCR from total RNA isolated from HEK 293 cells transfected with the three plasmids. As shown in Fig. 1, an amplicon of approximately 1.3 kb was obtained both from cells transfected independently with the three plasmids, and from plasmid pGEX-2T/P48 used as a positive control for PCR (Fig. 1A). However, as we attempted to detect P48 in HEK 293 cell lysates by immunoblotting, we were able to clearly observe the expected protein only in immunoblots of cells transfected with pVAX1/P48 (Fig. 1B). DNA sequencing confirmed the correct cloning of p48 in pVAX1. pVAX1/P48 was therefore chosen for nucleic acid immunizations.

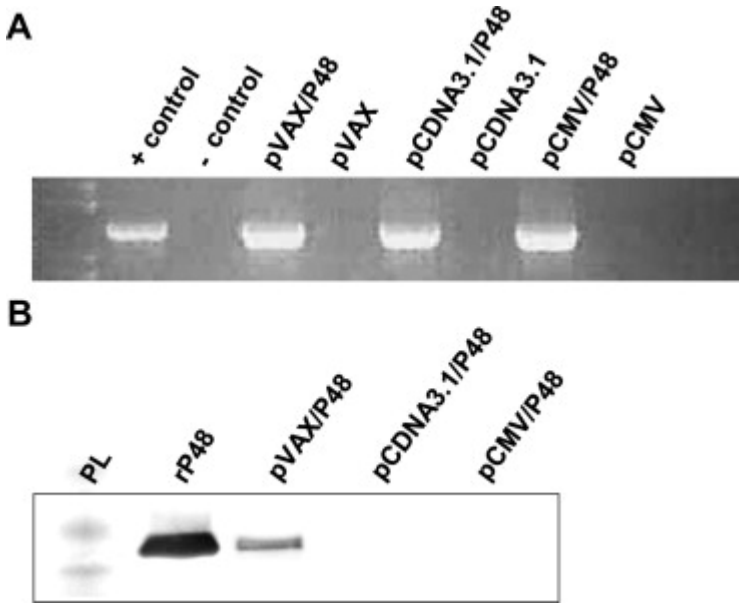

Fig. 1.

Transcription of P48 gene (A) and P48 protein expression (B) as detected by RT-PCR and immunoblotting in transfected HEK 293 cells. Cells were transfected with different P48 expression plasmids (and with the relative negative controls) as described in materials and methods. Total RNA was purified from transfected cells and amplified by RT-PCR with P48-specific primers. Production of specific mRNA (A) was assessed 48 h later in cells transfected with all the three plasmid tested. P48 protein was detected by immunoblotting (B) using a rabbit hyperimmune antiserum raised against P48 only in cells transfected with pVAX1/P48. Recombinant P48 was used as a positive control for immunoblotting. PL- Invitrogen BenchMark™ Pre-stained Protein Ladder (only the 40 kDa and 50 kDa bands are shown). PL: protein ladder.

3.2. Evaluation of humoral immune responses by pVAX1/P48 vaccination

The humoral immune responses induced by pVAX1/P48 vaccination were analyzed in mice by immunoblotting and rP48-ELISA. As shown in Fig. 2A, P48-specific IgG antibodies were only detected in pooled sera taken from vaccinated mice at Tf. Pooled sera of mice taken from the control group at the same sampling time never reacted with rP48. We tentatively used ELISA to clarify the IgG subclass and quantify antibodies (Fig. 2B–C). No statistically significant difference of IgG titers was detected between

sera taken at T0, T1, T2, T3 from control mice, whereas IgG titers of vaccinated mice at Tf were significantly different from titers of sera taken from the same mice at T0. However, when sera of vaccinated mice taken at different sampling times were tested for the presence of different antibodies subclasses, we failed to detect a statistically significant increase of IgG2a levels while still detecting a statistically significant increase of IgG1 levels. It should be noted that, a progressive increase of the IgG2a absorbances medians in vaccinated mice (even if not statistically supported) was observed.

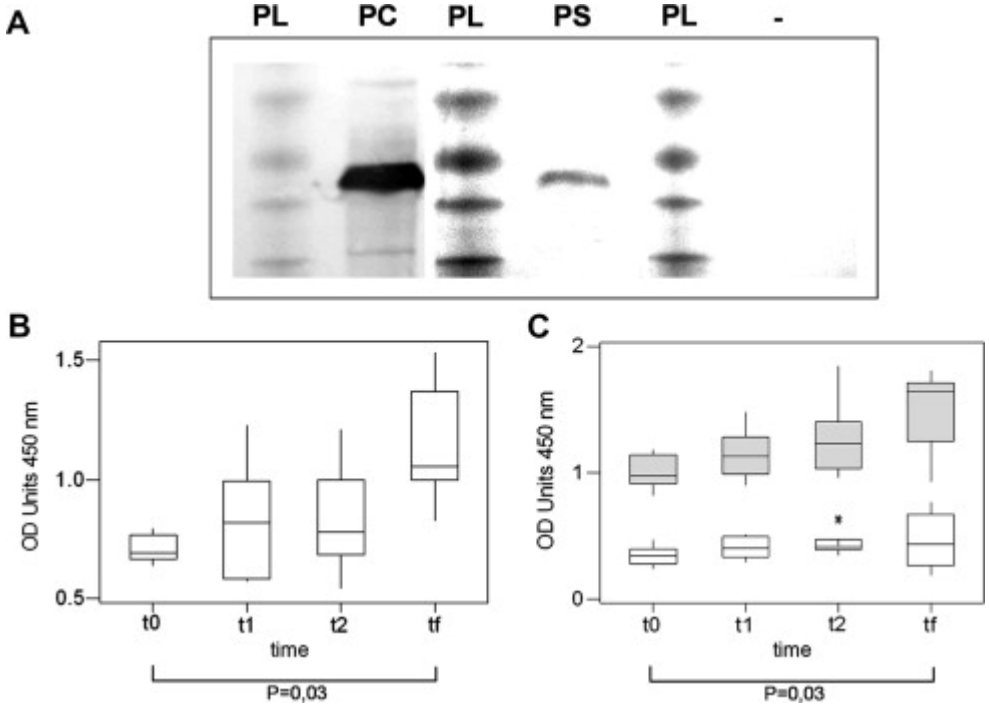

Fig. 2.

Evaluation of humoral responses in mice vaccinated with pVAX1/P48 by immunoblotting (A) and rP48-ELISA (B, C). In the immunoblots (A), pooled sera of vaccinated mice (PS) collected at T3 specifically reacted with rP48, whereas pooled sera taken from control mice (−) were always negative. PC = reactivity of rP48 against rP48 rabbit hyperimmune antiserum, used as a positive control. rP48-ELISA was used to evaluate IgG (B), IgG1 (dark gray boxes in C), and IgG2a (white boxes in C) levels in vaccinated mice. A significant increase of total IgG (P = 0.03) and IgG1 (P = 0.03) could be detected by rP48-ELISA only in vaccinated mice. An increase of IgG2a was also observed but was not statistically significant. Boxplots and P values were obtained by using the Anova and Mann-Whitney tests, respectively. PL = protein ladder.

3.3. Proliferation of mononuclear cells in the spleen

To determine whether P48-specific proliferation responses were induced in the immunized animals, the proliferation of mononuclear cells in the spleen was measured. Mononuclear cells from the spleens were harvested from mice eight weeks after the first inoculation with pVAX1/P48 (or with pVAX1, alternatively), and were stimulated with purified P48 protein for 72 h. As shown in Fig. 3, P48-specific proliferation in mononuclear cells of the spleen was efficiently induced by pVAX1/P48 vaccination. The mean stimulation index was estimated to be 4.8 in mice injected with pVAX1/P48. By contrast, no proliferation of mononuclear cells was found in pVAX1-immunized mice. Proliferation was never observed in experiments where Mycoplasma Capricolum subsp. capricolum P60 was used to stimulate mononuclear cells of the spleen (data not shown).

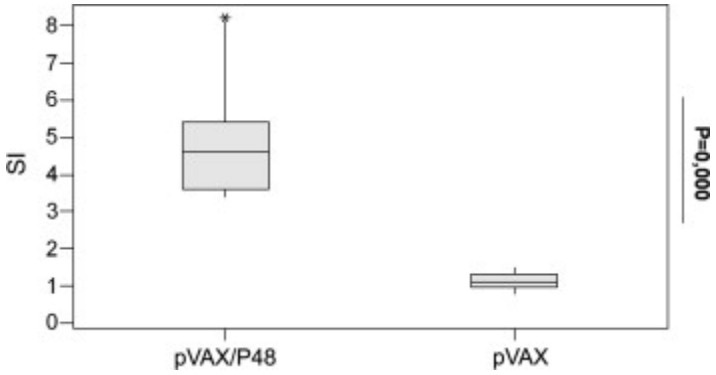

Fig. 3.

Proliferation of mononuclear cells in the spleen of mice injected with either pVAX1 or pVAX1/P48 after stimulation with P48. The mean stimulation index (SI) of vaccinated mice (injected with pVAX1/P48) was nearly 5 times greater then mean SI of control mice (Injected with pVAX1 only). Boxplots and P values were obtained by using the Anova and Mann–Whitney tests, respectively.

3.4. Cytokine-specific mRNA in mononuclear cells

To assay cytokine-specific mRNAs released from mononuclear spleen cells 8 weeks after the first immunization with pVAX1/P48, cytokine mRNAs were analyzed by reverse transcription real time PCR. As shown in Fig. 4, spleen cells of mice injected with pVAX1/P48 synthesized amounts of IFN-γ, IL-12, IL-2 mRNAs significantly higher than the same cells taken from mice injected with the vector pVAX1 only. A slight increase of T-bet, IL-4, and GATA-3 was also observed in mice immunized with pVAX1/P48.

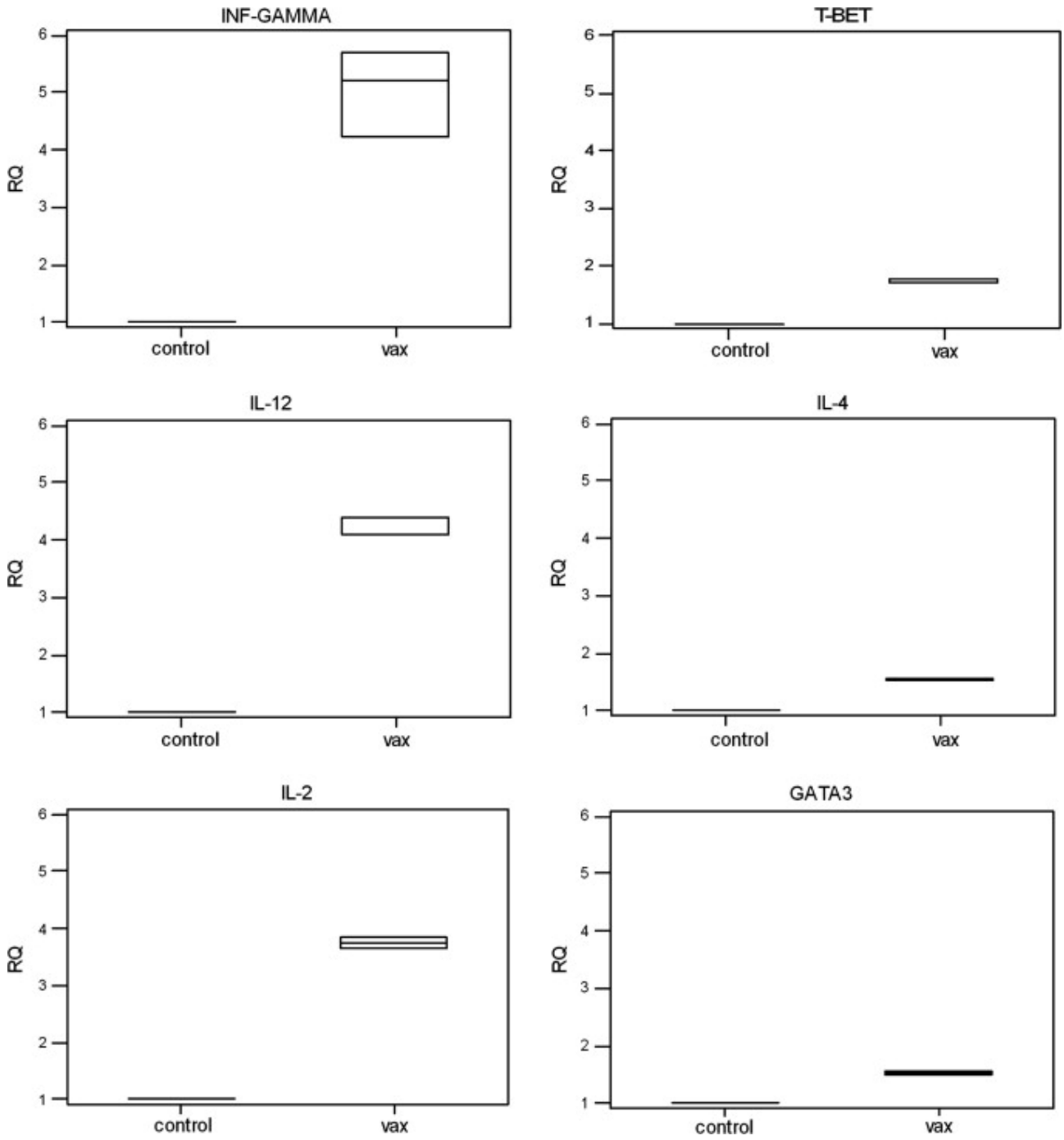

Fig. 4.

Relative quantity (RQ) of cytokines expressed in spleen of mice injected with pVAX1 (control), or pVAX1/P48, as evaluated by reverse transcription real-time PCR. RQ of transcription factors T-bet and GATA-3 are also given. Vaccinated mice produce significantly higher levels of cytokines respect to mice injected only with the vector pVAX1. Th1 cytokines, such as IFN-γ, IL-12, IL-2 were predominantly expressed.

4. Discussion and conclusions

The control of M. agalactiae through vaccination is an increasing challenge. Although several inactivated vaccines have provided protection to Ca (Tola et al., 1999, Greco et al., 2002 and Nicholas, 2002), they carry intrinsic disadvantages (e.g. DIVA diagnostics hampering, selection and use of adjuvants, requirements for multiple injections), and only produce a short-lived humoral immune response. Vaccination with plasmid DNA is an active area of investigation that is being applied to cancer and microbial pathogens associated with infectious diseases (Lowe et al., 2006). DNA vaccines have been reported to

persist in muscle for over one year, they induce effective immune responses with bacterial antigens, and are simply produced (Wolff et al., 1992 and Gurunathan et al., 2000). Recent investigations suggested that the genetic vaccination approach may induce both humoral and cellular immunities and represent a potentially new approach to design vaccines against mycoplasmas (Barry et al., 1995, Chen et al., 2003, Lai et al., 1997 and March et al., 2006).

In this study, a pVAX1-based plasmid expressing the P48 of M. agalactiae was designed and evaluated. As shown in Fig. 1, P48 mRNA was observed in transfected HEK 293 cells, and the P48 protein level was high enough to be detected by Western blotting. The production of P48-specific antibodies and the proliferation of spleen cells in pVAX1/P48-immunized mice (Fig. 2 and Fig. 3) further confirmed that P48 was expressed and induced significant immune responses in the immunized animals.

CpG motif acts as a “danger signal” and as a Th1 immune response enhancer in DNA vaccination through interaction with TLR9-positive cells (Liu et al., 2005). In addition to the CpG motifs contained in pVAX1, pVAX1/P48 contains two CpG motifs, ggCGtt and aaCGct, located in the p48 gene sequence, while hexameric CpG motifs which may inhibit the immunogenicity of DNA vaccines (Krieg et al., 1998 and Stratford et al., 2001) are absent in the same region.

P48 is an immunodominat invariable lipoprotein exposed on the membrane of M. agalactiae and belongs to the basic membrane protein family. The p48 gene (Rosati et al., 1999) and its homologues in Mycoplasma bovis (Robino et al., 2005), M. capricolum subsp. capricolum (Alberti et al., 2007), and Mycoplasma mycoides subsp.mycoides LC (unpublished data) are located upstream the ATP binding protein of an ABC transporter. P48 shares significant homology with several solute binding proteins of ABC transporters found in mycoplasmas and in other bacterial species. Indeed, P48 shares strong levels of homology with the ABC transporter Xylose binding protein of Mycoplasma pulmonis (E value 8e-30), Mycoplasma hypneumoniae (E value 1e-27), and Mycoplasma synoviae (E value 3e-22), and with solute binding proteins of uncharacterized ABC-type transport systems of Oenococcus oeni (E value 5e-08), Clostridium sticklandii (E value 5e-07), and Spiroplasma citri (E value 9e-08). These data indicate a putative function as solute binding protein also for P48 of Mycoplasma agalactiae. It has been shown that ABC transporters are highly immunogenic and represent ideal targets for the development of antibacterial vaccines. (Garmory and Titball, 2004). As an example, immunization with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory and systemic infection with Streptococcus pneumoniae (Brown et al., 2001 and Jomaa et al., 2006). Therefore P48, as a putative solute binding protein, may confer additional immunostimulatory effects and may represent an ideal candidate for the development of a DNA vaccine against M. agalactiae.

DNA vaccination has been demonstrated to induce both humoral and cellular immune responses. Th1 cells can activate macrophages to destroy intracellular microorganisms more efficiently and also activate B cells to produce strongly opsonizing antibodies such as IgG2a and IgG2b in mice (Gurunathan et al., 2000). The main effector function of Th2 cells is to drive B cells to proliferate and produce antibodies such as IgG1 and other types. A significant but weak increase of IgG in mice vaccinated with pVAX1/P48 was observed, apparently associated to the IgG1 subclass. We failed to observe an increase of IgG2a by rP48-ELISA. It was shown (Laylor et al., 1999) that priming and boosting of mice with the DNA vector pEE6DS-hCGß, expressing sequences encoding a transmembrane version of the b-chain of human chorionic gonadotropin (hCGb), was not followed by detection of appreciable levels of specific antibody. However, subsequent challenge with hCG protein in Ribi adjuvant elicited a strong and rapid secondary immune response. This response was of comparable magnitude to that produced following priming, boosting and challenge with protein in adjuvant. Therefore, DNA vaccination favours memory rather then effector B cell responses.

According to this, and to the levels of antibodies detected in vaccinated mice, pVAX1/P48 may have efficiently primed the humoral immune response.

Proliferation of CD4+ cells and cytokines expression profiles in the spleen, are typical of a Th1 immune response. Indeed high levels of IFN-γ, IL-12, and IL-2 were detected in spleens of immunized mice. A slight increase of IL-4 was also observed in the same samples. The two transcription factors T-bet and GATA-3, associated to Th1 and Th2 modulation, respectively, were both weakly activated in mice immunized with pVAX1/P48.

In conclusion we present evidence that DNA vaccination with pVAX1/P48 may induce specific Th1 and Th2 responses and represent a potentially new approach to design vaccines against M. agalactiae. We can not obviously make general predictions based on the immune responses of mice, and the potential of using pVAX1/P48 immunization for the control of contagious agalactia will be evaluated in sheep. The immunization (and challenge) of sheep will take into account the enhancement of humoral immunity through the prime-boost strategy (Dale et al., 2006), that is, priming with pVAX1/P48 and boosting with rP48.

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